Efficient VEGF gene expression using hypoxia-inducible neuron-specific vector system

Yeomin Yun

Department of Medical Science

The Graduate School, Yonsei University

Efficient VEGF gene expression using hypoxia-inducible neuron-specific vector system

Directed by Professor Yoon Ha

The Master's Thesis submitted to the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Master of Medical Science

Yeomin Yun

December 2014

This certifies that the Master's Thesis of
Yeomin Yun is approved.

Thesis Supervisor : Yoon Ha

Thesis Committee Member#1 : Keung Nyun Kim

Thesis Committee Member#2 : Jin Woong Bok

The Graduate School
Yonsei University

December 2014

ACKNOWLEDGEMENTS

2년이라는 시간이 지나 석사 학위 논문을 완성하기 까지 부족한 저를 격려해주시고 지도해주신 하윤 교수님, 김긍년 교수님, 실험에 많은 도움을 주신 한양대학교 이민형 교수님, 아낌없는 조언을 주신 복진웅 교수님께 감사의 말씀을 드리고 싶습니다. 또한 같은 실험실에서 저의 부족한 점에 대해 항상 도움을 주신 오진수, 차려화, 이혜영, 유영상 선생님과 함께 실험하며 많이 배울 수 있었던 이혜란 선생님, 처음 저에게 많은 것을 가르쳐주신 곽소정 선생님께 감사 드립니다. 제가 고민이 있을 때마다 항상 선배로서 좋은 충고와 이 공부를 시작할 수 있도록 이끌어준 선배 애리언니에게도 감사의 인사를 하고 싶습니다. 무엇보다 제가 하고 싶은 공부를 할 수 있도록 격려해주신 가족들에게도 깊은 감사의 말씀을 드리며 이 글을 마칩니다.

2014년 12월
윤여민 드림

TABLE OF CONTENTS

III. RESULTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Efficient VEGF gene expression using hypoxia-inducible neuron-specific vector system

Yeomin Yun

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Yoon Ha)

Since neurons play a critical role in the central nervous system, neuron-target gene expression system is important for gene therapy. Ischemic neurons following injury affect disruption of neurovascular unit which is mediated by astrocyte. Also reactive astrocyte contributes to astrogliosis that aggravate traumatic environment. Therefore, controllable gene expression system that expressed in neurons selectively more than glial cells and other cell types is necessary. We used neuron-specific enloase (NSE) promoter to target neurons. NSE is one of glycolytic isoenzymes and abundant in adult brain neurons due to their characteristics of expression in matured neurons. Because NSE is expressed high in various neuronal cell types, we can design neuron-target gene expression system using NSE promoter. Also, to improve gene expression under hypoxic ischemic environment like spinal cord injury (SCI), the combination of erythropoietin (Epo) enhancer and NSE promoter was used. Vascular endothelial growth factor (VEGF) is an angiogenic peptide and has neuroprotective effects as well as angiogenesis. VEGF is considered good

therapeutic gene because it protects injured neurons and promotes sprouting of blood vessels to support cell survival. In this study, the use of NSE promoter increases the expression of luciferase reporter gene and VEGF gene. The luciferase and VEGF gene expression is the highest with the plasmid vector including Epo enhancer under hypoxic conditions and also showed proliferation effect. With this hypoxia-inducible neuron-specific gene expression system, applied gene or cell therapy is promising to regenerate SCI.

Key words : Neuron-specific enolase, neuron promoter, erythropoietin, hypoxia-inducible, tissue-specific, vascular endothelial growth factor

Efficient VEGF gene expression using hypoxia-inducible neuron-specific vector system

Yeomin Yun

Department of Medical Science

The Graduate School, Yonsei University

(Directed by Professor Yoon Ha)

I. INTRODUCTION

Physical and mechanical injury to spinal cord results in neurologic disability and complete loss of function. Following traumatic primary spinal cord injury (SCI), a cascade downstream of events occur called secondary injury. Secondary injury including ischemia, neuronal apoptosis, demyelination, scar formation exacerbates injury severity during excitotoxicity, imflammatory response, free radical production.[1-3] Posttraumatic ischemic response is caused by hemorrhage, vasospasm, thrombosis of blood vessels[1] and develops microvascular damage in spinal cord. Neuron doesn't directly contact blood vessels but sensitive enough to ischemic condition and vulnerable.[4] Hypoxia is an important component in SCI treatment and related to blood vessel condition, vascular endothelial growth factor (VEGF) has been proposed a potent

therapeutic gene due to angiogenesis its original function. Angiogenic effect of VEGF supports neuron survival by restoring blood supply.[5,6] VEGF has neurotrophic and neuroprotective activity so that promote cell survival and blood vessel formation through high-affinity binding with phosphotyrosine kinase receptor.[7] Since VEGF is induced to express by hypoxia-inducible factor-1 (HIF-1) which is activated in ischemic condition,[8] ischemia should be necessary for VEGF expression. However, ischemic conditions following SCI trigger reperfusion that causes injury.[9] Although VEGF has various therapeutic effect, VEGF expression in normal tissue is associated with tumor formation.[10] At the injury site, reactive astrocyte induces astrogliosis that intensified SCI mediated by expressing VEGF. With ischemic neuron following injury, VEGF presents negative effect that result in disruption of neurovascular unit.[11] For these reasons, therapeutic VEGF expression should be regulated in ischemic neuron not normal tissue or non-neuronal cells. In order to express VEGF in neurons, we used neuron-specific promoter system and regulated in transcriptional level. Neuron-specific enolase (NSE) is one of glycolytic enzymes and abundant in matured neuron.[12] NSE level is very low in embryonic brain and appears in accordance with neurogenesis. Its endogenous mRNA and protein is higher in differentiated state and rare in other cells, undifferentiated neurons.[13,14] On the other hands, NNE level is high in embryonic brain and decrease with neuronal maturation. For these reasons, NSE is used as a marker protein of matured neurons. Using these characteristics, NSE promoter can be useful tool for target neuronal cells. In order to overexpress therapeutic gene in hypoxic condition and avoid express in normal tissue, erythropoietin (Epo)

enhancer was used. Epo gene is induced in hypoxic condition and Epo enhancer is well known for specific expression under hypoxic condition.[15,16] In this study, we constructed Epo enhancer-NSE promoter combined vector system. Using luciferase reporter gene, we evaluated hypoxia-inducible neuron-target gene expression *in vitro* and *in vivo*. Furthermore, substituted VEGF was used to test gene expression.

II. MATERIALS AND METHODS

1. Plasmid construction

pSV-Luc, pNSE-Lus, pEpo-SV-Luc, pSV-VEGF-pGL3-promoter was kindly provided by professor Minhyung Lee of Hanyang University. Epo cDNA was amplified by PCR (Hotstart pfu PCR PreMix, bioneer, Daejeon, Korea) using pEpo-SV-AP (Lab DNA stock). Two Epo fragments are used to construct pEpo-NSE-Luc. First, the primers for PCR are 5'- MluI-Epo, 3'- NheI-AscI-Epo (Table 1). Amplified one Epo enhancer was inserted into the MluI and NheI site upstream of NSE promoter. Second PCR of Epo enhancer was conducted using primers 5'- KpnI-SpeI-EcoRV-Epo, 3'- SacI-PmeI-Epo. PCR products of Epo were inserted into KpnI and SacI.

To construct pSV-VEGF, pNSE-VEGF, pEpo-NSE-VEGF, luciferase gene was removed from pSV-Luc, pNSE-Luc, pEpo-NSE-Luc. VEGF fragment including poly-A from pSV-VEGF-pGL3-promoter was introduced to luciferase location at HindIII and BamHI site. The plasmids were amplified in E.coli DH5α and purified using plasmid maxi prep kit (Qiagen, Hilden, Germany). The quantity of plasmids was measured using nanodrop (ND-1000, Thermo Fisher Scientific, Wilmington, DE, USA) by measuring the absorbance of plasmids at 260nm. The ratio of absorbance at 260nm and 280nm was used to assess the purity of DNA

Table 1. Primers and PCR conditions for plasmids construction

primer	sequences		
5'- MluI-Epo	CCCACGCGTGCCCTACGTGCTGTC		
3'- NheI-AscI-Epo	AAAGCTAGCGGCGCGCCCGCCGGTAGGT		
5'- KpnI-SpeI-EcoRV-Epo	TAT GGTACC ACTAGT GATATC GCCCT ACGTG		
3'- SacI-PmeI-Epo	AAAGAGCTCGTTTAAACCGCCGGTAGGT		
step	**temperature**	**time**	**No. of cycles.**
Pre-incubation	95℃	5 min	1
Denaturation	95℃	30 sec	35
Annealing	65℃	30 sec	
Extension	72℃	1 min	
Final-Extension	72℃	5 min	1

2. In vitro transfection

The mouse neural stem cells (mNSC; CRL-2925, ATCC, Manassas, VA, USA) were maintained in Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12 (DMEM／F-12; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Scientific HyClone, Logan, UT, USA), 1% penicillin streptomycin (P/S; Gibco, Grand Island, NY, USA). The mNSCs were seeded at a concentration of $1x10^5$/well into 6-well plates. Cells were cultured under normoxia conditions at 37℃ in 5% CO_2. After 24 hours, plasmids (2μg/well) were transfected with polyethylenimine (PEI, 25000 D; Sigma Aldrich, St. Louis, MO, USA). PEI is a gene delivery vector for plasmids transfection. PEI/cDNA complexes were prepared at the ratio of 5:1 N/P and added to cells with serum-free medium for 4 hours at 37℃ in 5% CO_2. After 4 hours, cells were cultured under normoxia (20% O_2) or hypoxia (1% O_2) conditions for 48 hours.

3. Cell differentiation

For differentiation, mNSCs were seeded into 6-well plates ($1x10^5$/well) 24 hours prior to treat retinoic acid (RA; Sigma, St. Louis, MO, USA) with culture medium. The culture medium was exchanged to differentiation medium containing DMEM/F12, 1% FBS, 1% nonessential amino acids (NEAA, Gibco, Grand Island, NY, USA), 0.5% P/S and 1μM RA. Neuronal differentiation is induced by 1μM RA for 7days. Differentiation medium was changed every two days. After 5 days, differentiated cells were transfected

and cultured for 48 hours under normoxia or hypoxia conditions. At that time, differentiation medium was exchanged to transfection medium (DMEM/F12, 1% P/S). After 2 days of transfection, cells were fixed with 4% paraformaldehyde (PFA; Millipore, Darmstadt, Germany) for immunocytochemistry or lysed with reporter lysis buffer (Promega, Madison, WI, USA) for luciferase assay.

For Pheochromocytoma 12 (PC12) cells differentiation, $3x10^5$/well was seeded into poly-L-lysine (Sigma Aldrich, St. Louis, MO, USA) coated 6-well plates. PC12 culture medium is Roswell Park Memorial Institute 1640 (RPMI 1640; Gibco, Grand Island, NY, USA) supplemented with 10% FBS, 1% P/S. After 24 hours, 100ng/μl nerve growth factor (NGF; R&D System, Minneapolis, MN, USA) was added with serum-free medium (RPMI 1640, 1% P/S). Differentiation medium was exchanged every 2 days. pDNA transfection was conducted after 5 days of treating NGF and cultured another 48 hours.

4. Luciferase assay

To compare the effect of promoter with or without enhancer, luciferase reported gene was used. Cells transfected with pSV-Luc, pNSE-Luc, pEpo-NSE-Luc were allowed to express luciferase in normoxia or hypoxia. After 48 hours and lysed with 300μl of reporter lysis buffer and undergo one freeze-thaw cycle. The lysates were transferred to a 1.5ml tube and centrifuged at 12000 rpm for 2 minutes. The supernatant lysates were transferred to a fresh 1.5ml tube and protein concentration was measured

using BCA kit (pierce, Rockford, IL, USA). Luciferase activity was determined by Microplate luminometer (Berthold, Bad Wildbad, Germany) in terms of relative light units (RLU) per milligram of total protein.

5. Luciferase expression imaging

In vivo imaging system (IVIS; Xenogen, Alameda, CA, USA) was used to confirm luciferase expression in mNSCs transfected pSV-Luc, pNSE-Luc. mNSCs were seeded into 6-well plates ($1x10^5$/well) 24 hours prior to transfection. After 48 hours plasmids transfection as previous protocol, IVIS was conducted using Living Image (Xenogen). Substrate D-luciferin (150μg/ml) was added and bioluminescence was measured after 2-3 minutes.

6. Reverse transcriptase polymerase chain reaction

Total RNA was isolated from normoxia or hypoxia cultured mNSCs using RNeasy Plus Mini kit (Qiagen, Hilden, Germany). The extracted RNA was treated with RNase-free water. The concentration of RNA was measured by measuring the absorbance at 260nm. Isolated RNA was converted to cDNA using RT PreMix (bioneer, Daejeon, Korea). The PCR amplification was performed with 1μg of cDNA as follows : preincubation (95℃ for 5 min), 35 cycles of PCR (95℃ for 30 sec, 59℃ for 30 sec, 70℃ for 30 sec) and extension (70℃ for 10 min). The specific primer sequences were VEGF 5'-CCCAAGCTTGAAACCATGAACTTGCT-3' (forward), 5'-GCTCTAGATCATTCATTCATCACCGCCT-3' (reverse). The PCR

products were separated using electrophoresis on 0.8% agarose gels.

7. Enzyme-linked immunosorbent assay

Cells were transfected with pNSE-VEGF, pEpo-NSE-VEGF under normoxia or hypoxia conditions. Cell culture supernates was collected in a 1.5ml tube and stored in -20℃ until used. The VEGF expression was measured by ELISA kit (Abfrontier, Seoul, Korea) in accordance with the manufacturer's protocol. Sample volume of 100μl was added to the designed wells precoated with VEGF specific monoclonal antibodies and incubated at 37℃ for 90 min. After discard the contents, added 0.1ml of biotinylated VEGF antibodies into each well and incubated at 37℃ for 60 min. Wash the plate three times with 0.01M PBS. Prepared ABC working solutions was added into each well and incubated at 37℃ for 30 min. After 5 times of wash the plate, 90μl TMB color developing agent was added and incubated 25℃ in dark for 20 min. After incubation period, TMB was catalyzed and changed into yellow color by TMB stop solution. The density of yellow was measured at 490nm.

8. Immunocytochemistry

For immunostaining, plates were coated with 0.1% gelatin for 1 hour. mNSCs were seeded in the density of $1x10^5$. Cells were differentiated for 5days and transfected with pNSE-Luc. For differentiation, RA concentration was diluted to 1μM, 0.1μM, 0.01μM gradually. Two days after transfection, cells were fixed with 4% PFA and washed with 0.3% TritonX-100. Fixed cells

were blocked with 10% NDS, 0.3% TritonX-100 in PBS at room temperature for 1 hour. Cells were immunostained using anti-Luc (1:1000, Sigma, St. Louis, MO, USA), anti-microtubule-associated protein-2 (MAP-2; 1:500, Abcam, Cambridge, UK) antibodies. Confocal images were obtained with confocal microscopy (Carl Zeiss LSM-700, Jena, Germany).

9. *In vitro* cell proliferation assay

mNSCs were plated in 96-well with a density of $2x10^3$/well and followed by incubation for 24 hours. pEpo-NSE-luc was transfected with the same protocol as previous state. Cells were incubated under normoxia or hypoxia conditions. Cell proliferation was measured by MTT assay every day at 24 hours interval. 5mg/ml MTT solution was added to each well, followed by 4 hours incubation at 37℃. The reaction was stopped by removing the MTT solution and added DMSO to dissolve formazan crystals. VersaMax ELISA Microplate Reader (Molecular Device, Sunnyvale, CA, USA) tested absorbance 490nm value.

10. Construction of spinal cord injury model

To investigate whether VEGF exhibits neuroprotective effect, plasmids were introduced to spinal cord injury site for gene therapy. The Animal Care and Use Committee of the Medical Research Institute of Yonsei University College of Medicine approved all protocols. All procedures were performed according to international guidelines on the ethical use of animals, and the

number of animals used was minimized. Laminectomy was performed at T9 level in adult male Sprague-Dawley rats (200-250g; OrientBio, Gyeonggi-do, Korea). After anesthesia (100mg/kg ketamine, Yu-han, Seoul, Korea), exposed spinal cord T9 level was injured by clip compression (40g; Biemer, Irvine, CA, USA) for 10 minutes. Plasmids (20μg) were directly injected into caudal to spinal cord injury site using Hamilton syringe (Hamilton, Reno, NV). Experimental animals were divided into four groups: PBS control (10μl), pSV-Luc, pNSE-Luc, pEpo-NSE-Luc, later, VEGF gene was used instead of luciferase gene.

11. Tissue extraction

Spinal cord tissues were obtained by heart perfusion with saline (pH 7.5) and 4% paraformaldehyde at 2 days after plasmids injection. Obtained tissues were homogenized with Pro-prep (iNtRON biotechnology, Gyeonggi-do, Korea) and lysed for 1 hour in ice. Lysed tissues were spin down and collect supernatant. Luciferase expression was measured as previous state in luciferase assay.

12. Statistical analysis

Data are analyzed using student's t-test to compare between two experimental groups. One-way anova was used to compare data among three experimental groups. P-value under 0.05 was considered to be statistically significant.

III. RESULTS

1. Construction of hypoxia-inducible neuron-specific gene expression plasmids.

For neuron-specific gene expression, we constructed pSV-luc, pNSE-Luc, pEpo-NSE-Luc (Figure 1). Unlike SV promoter that commonly used in expression vector [17,18], NSE promoter could achieve transcriptional regulation in neuronal cells. Expression efficiency of vector including SV promoter or NSE promoter was assessed with the expression level of luciferase reporter gene. In addition to tissue targeting promoter, Epo enhancer was used to expression. Because of ischemic microenvironment following spinal cord injury, hypoxia inducible gene expression system increases the luciferase expression. Luciferase fragment from pSV-Luc, pNSE-Luc, pEpo-NSE-Luc was further replaced the VEGF therapeutic gene, leading to the construction of pSV-VEGF, pNSE-VEGF, pEpo-NSE-VEGF.

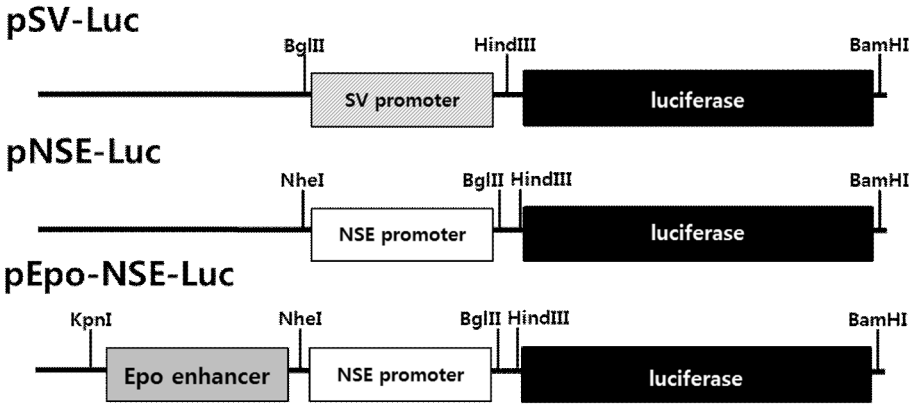


Figure 1. Structure of pSV-Luc, pNSE-Luc, pEpo-NSE-Luc plasmids. The pSV-Luc contains simian virus 40 promoter. The pNSE-Luc contains neuron-specific enolase promoter. The pEpo-NSE-Luc has two copies of Epo enhancer upstream of NSE promoter.

2. Luciferase expression in various neuronal cells.

In vitro transfection assay was performed to compare the promoter activity of pSV-Luc, pNSE-Luc. The plasmids were transfected into mNSCs, N2a, C6 cells. pDNA/carrier complexes are prepared at the N/P ratio of 5 which is optimal to gene transfer with low toxicity. We transfected three groups including control, pSV-Luc and pNSE-Luc. Control group is transfected only carrier PEI with the same concentration. In mNSCs, luciferase expression with NSE promoter was higher than with SV promoter which has moderate expression characteristics (Figure 2A, B). N2a cells had the same luciferase activity (Figure 2C) that pNSE-Luc showed two folds increases compared to pSV-Luc. However, in C6 glioblastoma cells, there was no difference of luciferase expression between pSV-Luc and pNSE-Luc (Figure 2D). From the results, we confirmed that NSE promoter induced high gene expression in various neuronal cells and could be used for tissue target promoter. Moreover NSE promoter is not restricted in neurons because it is also active in neural stem cells.

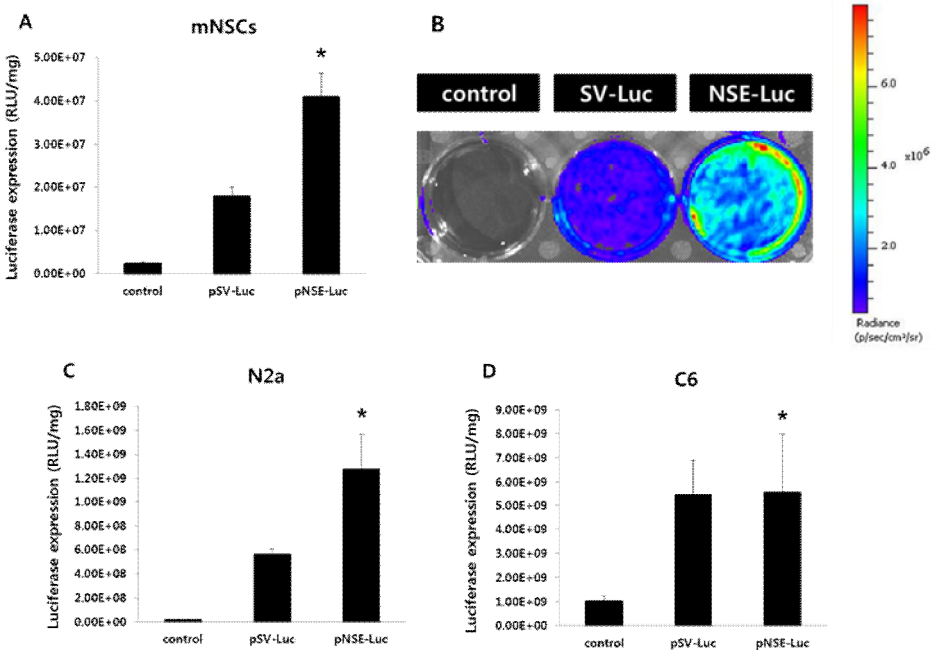
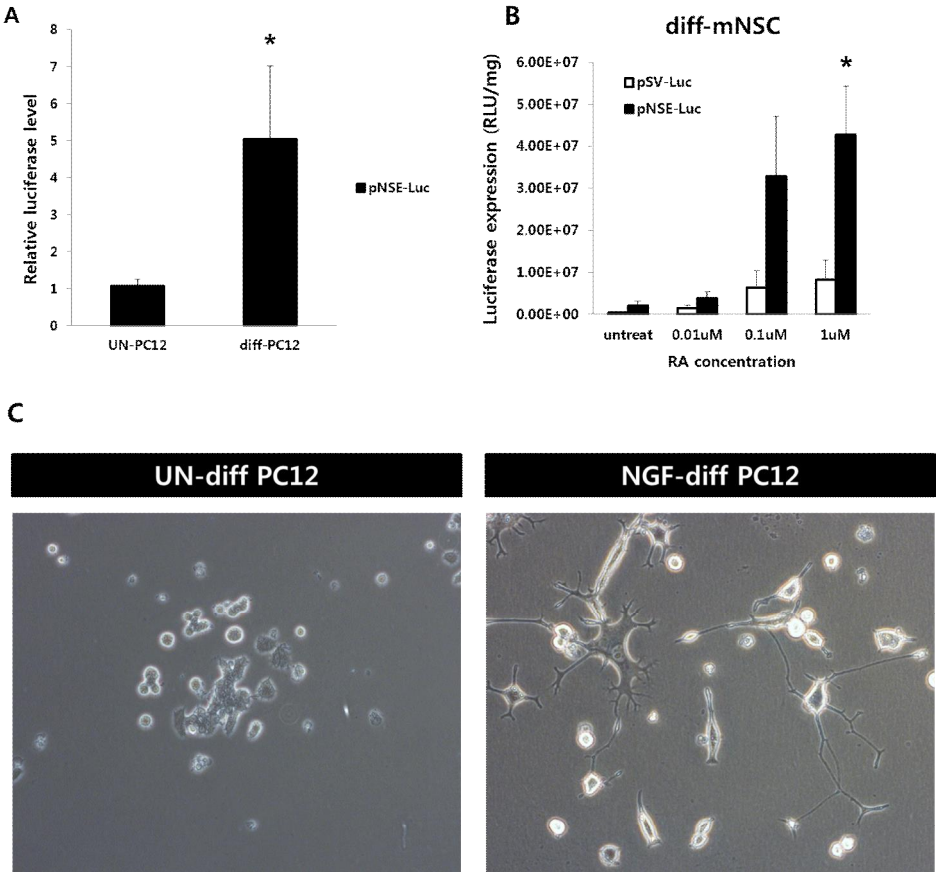


Figure 2. Luciferase expression in various neuronal cells. Plasmids were transfected into mNSCs (mouse neural stem cell), N2a (neuroblastoma cells), C6 (glioblastoma cells). Control means transfected only carrier PEI. pDNA/carrier complexes were prepared at a 5:1 N/P ratio. Plasmids transfected cells were incubated for 48 hours in normoxia conditions. Luciferase level was measured by luciferase assay. pNSE-Luc transfected group showed 2 folds higher expression level than pSV-Luc group in both mNSC (A) and N2a (C). (B) Transfected mNSCs IVIS image. However, C6 glial cells showed similar expression level in pSV-Luc and pNSE-Luc transfected groups (D).

3. Luciferase expression in differentiated neurons.

NSE promoter is responsible for high expression level in differentiated neurons because NSE appears during neurogenesis and abundant in matured neurons.[19] NSE protein is produced originally in neuroendocrine and its endogenous mRNA level is higher in differentiated PC12 neuroendocrine cells than undifferentiated PC12 cells.[12,14] PC12 cells were differentiated using nerve growth factor (NGF) [20] and confirmed the neurite outgrowth (Figure 3C). In NGF-differentiated PC12, luciferase expression level induced by pNSE-Luc was higher than un-differentiated PC12, approximately 5 folds (Figure 3A). Because NSE is correlated with the differentiation state of neuronal cells, we measured the luciferase activity of gradually differentiated mNSCs treated the retinoic acid. 1μM of retinoic acid is optimal concentration [21] for differentiation and diluted to 0.1μM, 0.01μM. Untreat means treated only DMSO due to retinoic acid is dissolved in DMSO to use. It takes 7 days until differentiation and has long branching processes morphological appearance (Figure 3D). 5 days after treating RA, we transfected plasmids into cells and allowed the luciferase gene 48 hours to express. Luciferase expression with NSE promoter in 1μM retinoic acid-differentiated mNSCs is much higher than control mNSCs (Figure 3B). In differentiated mNSCs treated 1μM retinoic acid, pNSE-Luc showed up to 5.2 folds higher luciferase expression than pSV-Luc, 20.4 folds higher than untreated-RA mNSCs. These results represent NSE promoter is more active in differentiated state.


A
Relative luciferase level
8
7
6
5
4
3
2
1
0
*
pNSE-Luc
UN-PC12
diff-PC12
B
diff-mNSC
Luciferase expression (RLU/mg)
6.00E+07
5.00E+07
4.00E+07
3.00E+07
2.00E+07
1.00E+07
0.00E+00
pSV-Luc
pNSE-Luc
*
untreat
0.01uM
0.1uM
1uM
RA concentration
C
UN-diff PC12
NGF-diff PC12

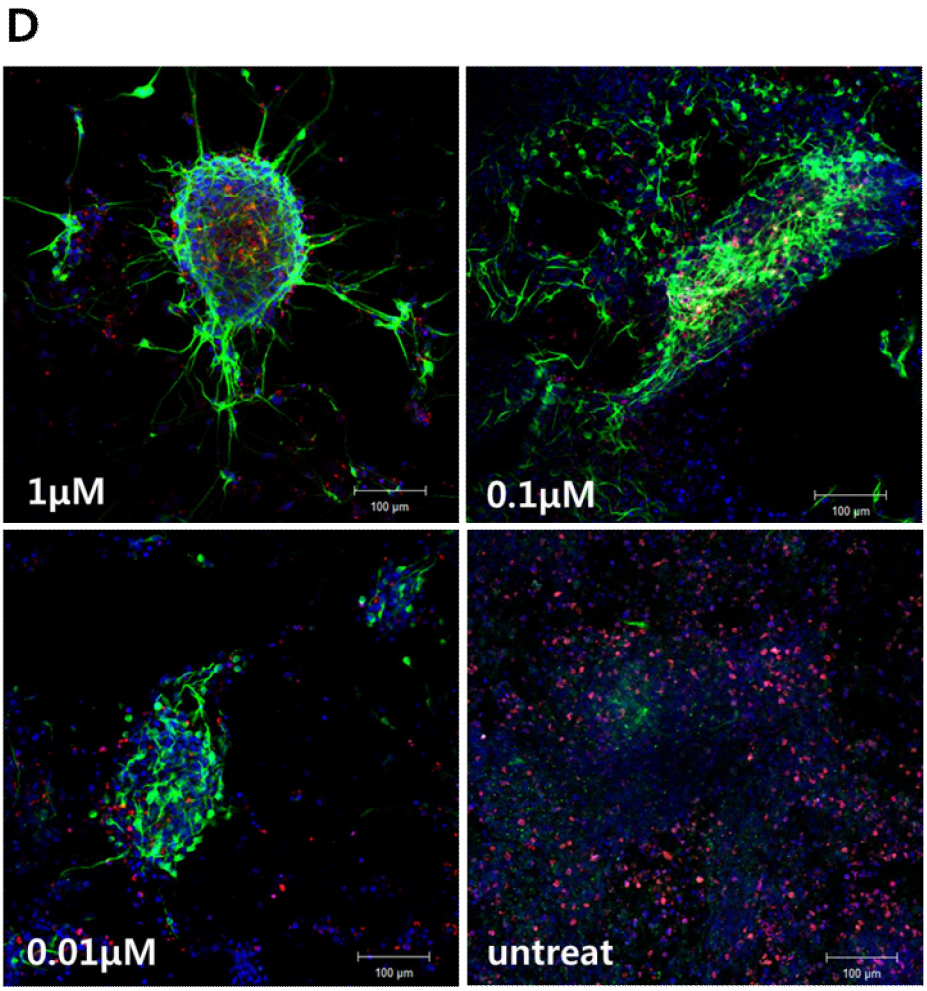


Figure 3. Luciferase activity of NSE promoter in differentiated cells. PC12 cells have the differentiation ability upon nerve growth factor (NGF). Differentiated PC12 has higher level of endogenous NSE mRNA and protein than undifferentiated PC12. Luciferase level of pNSE-Luc transfected differentiated PC12 was 5 folds higher than undifferentiated PC12 (A). Morphological appearance after 7 days of treating NGF (C). Differentiation of mNSCs was induced retinoic acid (RA). Immunostaining of mNSCs treated 1μM, 0.1μM,

0.01μM gradually and untreated RA. anti-Luc (1:1000, sigma), anti-microtubule-associated protein-2 (MAP-2, 1:500, abcam) antibodies was used (D). Since NSE protein is abundant in matured neurons, luciferase level in differentiated mNSCs treated 1μM RA was 20.4 folds higher than untreated-RA mNSCs (B). NSE promoter activity was also 5.2 folds higher compared to SV promoter in 1μM RA-differentiated mNSCs.

4. Luciferase expression under normoxia and hypoxia conditions.

To increase gene expression under ischemic microenvironment following injury, hypoxia inducible gene expression system was constructed. To regulate gene expression transcriptionally, promoter and enhancer was used to control gene expression.[22] Epo gene is induced under hypoxia conditions.[15] In addition to neuron target promoter, Epo enhancer was used to hypoxia-inducible neuron-specific dual effects. To construct pEpo-NSE-Luc, Epo enhancer was inserted into upstream of NSE promoter. pNSE-Luc, pEpo-NSE-Luc was transfected into mNSCs and cultured under normoxia or hypoxia conditions for 48 hours. Luciferase expression level of plasmids including Epo enhancer was 3.2 folds higher than plasmids without Epo enhancer in hypoxia conditions (Figure 4A). Compare to pNSE-Luc which is absent Epo enhancer, pEpo-NSE-Luc also showed approximately 3.4 folds of luciferase expression. Moreover, pEpo-NSE-Luc transfected group represents similar gene expression level as pNSE-Luc transfected cells in hypoxia. It suggests that gene expression was regulated to express in hypoxia condition and limited in normal condition. To confirm the pEpo-NSE-Luc activity in differentiated neurons, mNSCs was treated 1μM RA for 7days. 5days after differentiation, pEpo-SV-Luc and pEpo-NSE-Luc was transfected. Luciferase level of pEpo-NSE-Luc is higher in differentiated mNSCs than undifferentiated state (Figure 4B). In differentiated mNSCs, pEpo-NSE-Luc showed 14.1 folds higher luciferase than pEpo-SV-Luc that only has promoter difference. Combination of Epo enhancer and NSE

promoter demonstrated dual specific effect in targeted-tissue under hypoxic conditions.

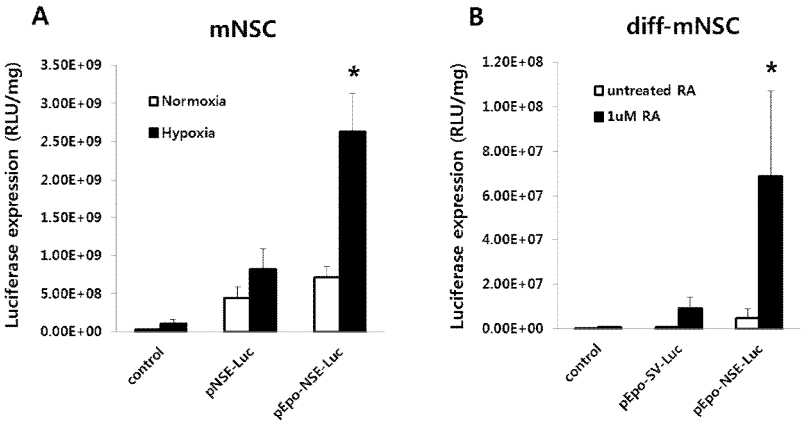


Figure 4. Luciferase level of pNSE-Luc, pEpo-NSE-Luc transfected mNSCs. Transfected cells were incubated for 48 hours under normoxia or hypoxia conditions. Plasmids including Epo enhancer increased luciferase expression compared to pNSE-Luc approximately 3.2 folds higher. pEpo-NSE-Luc transfected cells showed 3.6 folds higher luciferase expression under hypoxia than normoxia conditions (A). (B) shows luciferase expression in differentiated and undifferentiated cells transfected pEpo-SV-Luc, pEpo-NSE-Luc. In differentiated mNSCs, pEpo-NSE-Luc transfected cells showed higher luciferase expression compared to pEpo-SV-Luc only difference in promoter.

5. VEGF expression under normoxia and hypoxia conditions.

VEGF is considered to have therapeutic effects that neuroprotection and angiogenesis. Through mitogen-activated protein kinase (MAPK) pathway, VEGF promotes the survival and growth of neurons.[7,23] In order to express VEGF gene using hypoxia-inducible neuron-specific vector, luciferase fragment of pNSE-Luc, pEpo-NSE-Luc was replaced VEGF gene (Figure 5). To confirm the expression of VEGF using dual specific vector system, RT-PCR is performed. pEpo-NSE-Luc transfected group showed the best amplified band under hypoxia conditions (Figure 6A). Epo enhancer increased the expression of VEGF gene under hypoxia conditions mimic ischemic injured microenvironment. Since VEGF is secretary growth factor, cell culture medium was collected and secreted VEGF is measured using VEGF antibody detection. VEGF expression level is higher in pEpo-NSE-Luc transfected hypoxic mNSCs (Figure 6B). The results suggested that the highest VEGF gene mRNA and protein in hypoxic conditions is the effect of Epo enhancer and NSE promoter activity.

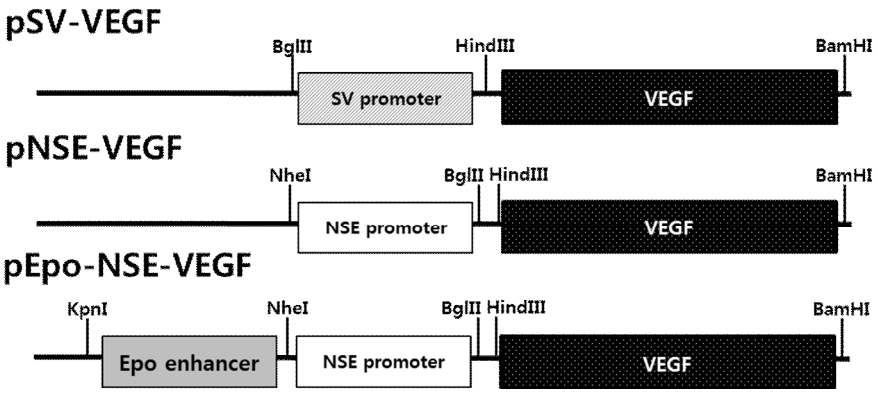


Figure 5. Structure of pSV-VEGF, pNSE-VEGF, pEpo-NSE-VEGF plasmids. Luciferase gene was substituted for VEGF gene from pSV-Luc, pNSE-Luc, pEpo-NSE-Luc plasmids using HindIII and BamHI restriction enzyme sites.

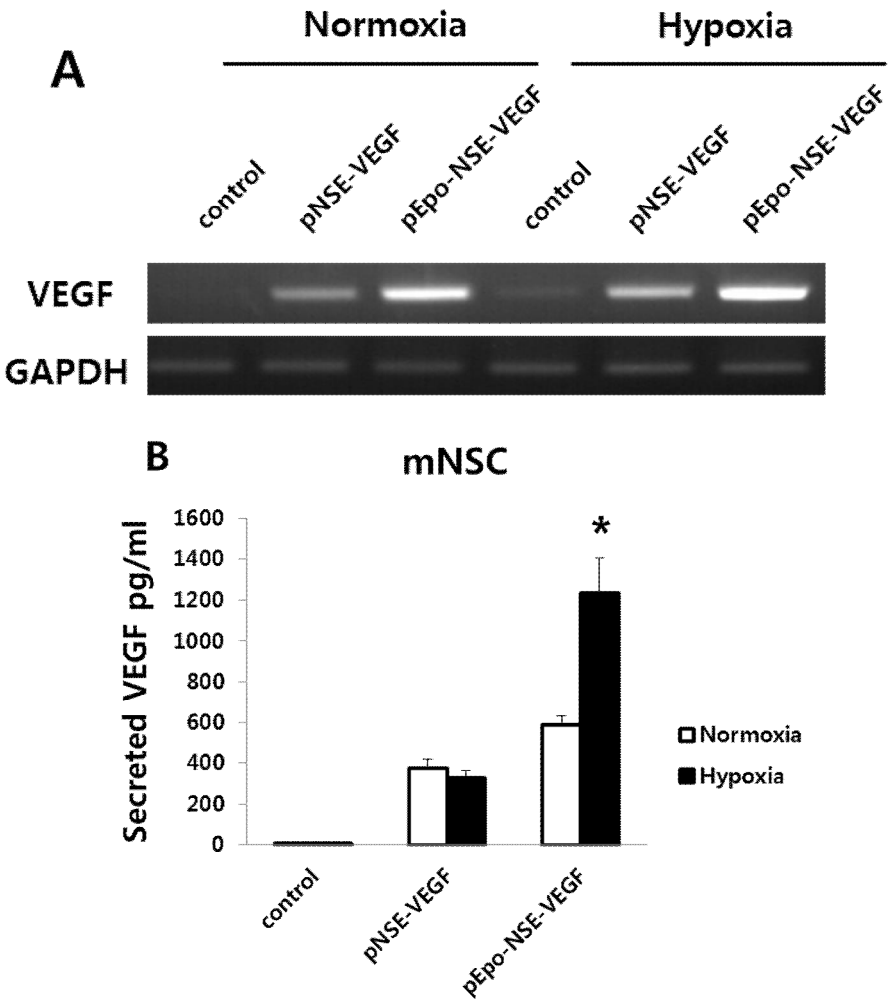


Figure 6. Gel electrophoresis after RT-PCR. Isolated RNA was converted to cDNA and amplified. pEpo-NSE-VEGF transfected mNSCs showed the best amplified band under hypoxic conditions (A). Using Enzyme-linked immunosorbent assay, secreted VEGF was measured (B). Like luciferase reporter gene, VEGF gene expressed higher under hypoxic conditions.

6. Gene expression in spinal cord injury models

To evaluate the expression level of luciferase gene *in vivo*, pSV-Luc, pNSE-Luc, pEpo-NSE-Luc was directly injected to spinal cord following injury. Construction of spinal cord injury model was performed as previous state in Materials and Methods. After 10 minutes of clip compression to T9 level, plasmids were directly injected into caudal to injury site at the N/P ratio of 5. After 48 hours, tissue was extracted and performed luciferase assay. The expression level of luciferase was the highest in pEpo-NSE-Luc group among four groups including control due to the role of Epo enhancer in ischemic condition of injured spinal cord. Expression level of pNSE-Luc was higher than pSV-Luc. There results demonstrated the activity of NSE promoter and Epo enhancer *in vivo*.

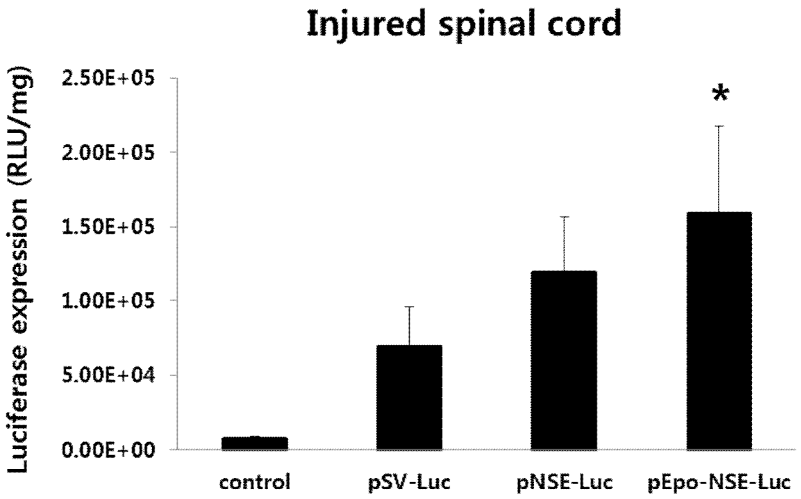


Figure 7. Luciferase expression in injured spinal cord. pDNA/PEI complex was directly injected to spinal cord following clip suppression for 10 minutes. pEpo-NSE-Luc showed the highest gene expression.

7. Proliferation effect of VEGF

Since VEGF is well known for neuroprotective effect, therapeutic effect was confirmed by evaluating cell proliferation *in vitro*. Cells were transfected pEpo-NSE-Luc and cultured under normoxia or hypoxia condition for 24, 48, 72 hours. Control group which is transfected only carrier showed no significant effect of proliferation for 72 hours. However, the absorbance value of cells at 490nm showed the higher level under hypoxia than normoxia (Figure 8).

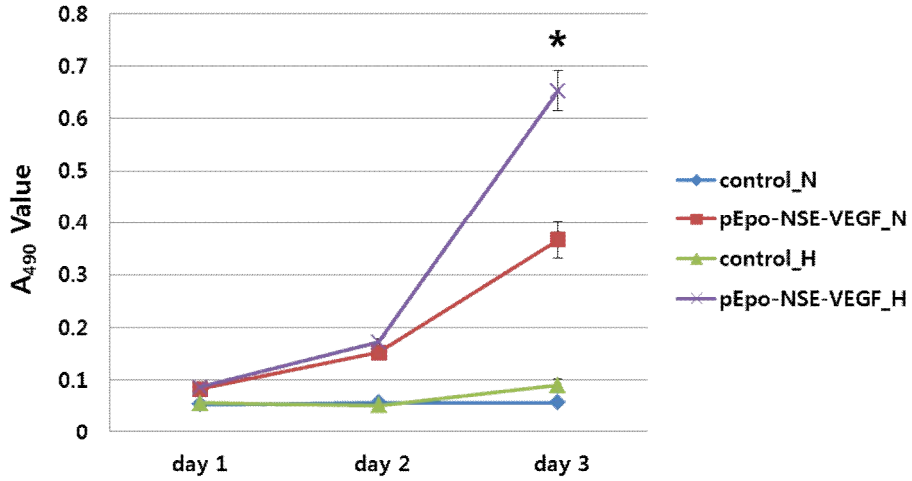


Figure 8. Proliferation effect of hypoxia-inducible neuron-specific VEGF. MTT assay determined cell viability under certain circumstances. pEpo-NSE-VEGF transfected cells proliferated up to 7.2-fold increase in hypoxia compared with 6.4-fold increase in normoxia by control contained only PEI carrier. (N : normoxia, _H : hypoxia)

IV. DISCUSSION

This study aimed specific expression of therapeutic gene in neuronal cells. In gene therapy, there are two ways to target the tissue. One is modifying viral vector to have tissue tropism. The other is using promoter to regulate gene expression in transcriptional level. To target the neuronal cells, we used NSE promoter. NSE protein is originally expressed in neuroendocrine and increased during neurogenesis. However, NSE promoter is active also in neural stem cells not only in matured neurons. It means NSE promoter can target various neuronal cells under developing stages. In order to test the capacity of neuron-specific promoter, we transfected pSV-Luc, pNSE-Luc to various neuronal cells. In mNSCs and N2a neuronal cells, pNSE-Luc showed higher luciferase expression than pSV-Luc. In contrast, luciferase activity introduced by pSV-Luc and pNSE-Luc showed no difference in C6 glial cells. PC12 cells that usually used for studying neuronal differentiation due to its ability to differentiate upon nerve growth factor [20] were transfected with pNSE-Luc. Differentiated-PC12 cells showed more than 5 times higher luciferase expression than undifferentiated-PC12 cells. In mNSCs, as cells differentiated upon retinoic acid concentration, so did the expression of luciferase. It showed the highest luciferase expression in 1μM RA treated mNSCs with pNSE-Luc transfection. In immunostaining, morphology of differentiated mNSCs aggregated and formed a mass. The number of neurites was increased and showed long neurite outgrowth.[21] Following confirm the NSE activity, Epo enhancer was inserted into the upstream of NSE promoter.

In order to increase the gene expression in ischemic injury site, we used Epo

enhancer which is induced under hypoxia condition. We constructed combined vector system pEpo-NSE-Luc to dual-specific gene expression. Epo is a hormone which regulates erythropoiesis. Epo synthesis is activated when exposed to low blood oxygen by binding of hypoxia-inducible factor-1 (HIF-1) to Epo gene enhancer. Therefore, Epo enhancer has been used to increase reporter gene by hypoxia induction.[15,16] pEpo-NSE-Luc transfected group showed the best expression under hypoxia conditions compared with normoxia or pNSE-Luc. In the case of differentiated mNSCs, luciferase level of pEpo-NSE-Luc transfected group is higher than undifferentiated mNSCs. Also, pEpo-NSE-Luc transfected group in both differentiated cells and undifferentiated cells showed higher luciferase expression compared with pEpo-SV-Luc which is only different in promoter. It means that overexpression in neuronal cells can take place at the injury site. Besides Epo enhancer, there are other ways to increase reporter gene under hypoxia such as pRTP801 promoter, oxygen-dependent degradation (ODD) domain and Epo 3'-untranslated region (UTR).[24-26] However, Epo enhancer has better activity and induced more expression in previous reports.[10,24-29] Also, enhancer is not limited in orientation and location unlike promoter.[22] Therefore, enhancer-promoter combined vector system could show dual specific effect. Instead of luciferase gene, VEGF therapeutic gene was cloning to downstream of Epo enhancer and/or NSE promoter. Amplified mRNA and secreted VEGF quantity was measured by RT-PCR, ELISA. pEpo-NSE-VEGF transfected group showed the highest expression level under hypoxia condition expectably.

VEGF is expressed in neurons, glial cells and has autocrine effect on neurons

and paracrine action through interaction between neurons and glial cells.[5] VEGF and VEGF receptor-2 are inducible under hypoxic condition and exert neuroprotective effect in response to hypoxia, oxidative stress through mitogen-activated protein kinase (MAPK) and phosphatidyloinositol 3'-kinase (PI3K)/Akt signal transduction system.[23,30] VEGFR-2 is also expressed on the surface of spinal cord *in vivo*.[31] Under stress condition like ischemia, oxidative stress, multiple molecular pathway lead to neuroprotective action by inhibiting apoptosis, stimulating neurogenesis.[32-34] VEGF induce vessel formation that restores blood oxygen delivery so that increases cell survival and proliferation.[35-37] However, exogenously delivered VEGF affect physiological impact in non-ischemic and normal tissue so that results in formation of vascular tumor.[38] Therefore, it is necessary to restrict gene expression in ischemic tissue to avoid side effect.

In addition to Epo enhancer which is inducible under hypoxia condition, using neuron target promoter makes it possible to express therapeutic gene in neurons. With this combined vector system, dual-specific gene expression can be a useful gene therapy tool.

V. CONCLUSION

Spinal cord injury occur a complex interaction between neuron and glial cells resulting in dysfunction. At the SCI site, neural precursor cells were predominantly differentiated into astrocyte rather than oligodendrocyte. Since reactive astrocyte exhibits negative effect in injury site, it is necessary to regenerate neuron specifically. To target the neuron, NSE promoter was used in this study. With neuron-specific promoter, reporter gene was expressed in neuronal cells not in glial cells. Also, it showed more gene expression depending on the differentiated state of mouse neural stem cells. It is contributed to that NSE is abundant in matured neuron. In SCI, ischemia is an important cause of secondary injury because neurovascular damage reduced oxygen level. Therefore, in addition to neuron-specific promoter, hypoxia-inducible vector would be increase gene expression. For that, Epo enhancer was used to combine NSE promoter. The results showed that Epo enhancer and NSE promoter dual specific vector increases cell proliferation and gene expression under hypoxic condition both *in vitro* and *in vivo*. Using this combined hypoxia-inducible neuron-specific promoter vector system, therapeutic gene can exert dual specific effect.

REFERENCES

1. Park E, Velumian AA, Fehlings MG. The role of excitotoxicity in secondary mechanisms of spinal cord injury: a review with an emphasis on the implications for white matter degeneration. J Neurotrauma 2004;21:754-74.
2. Lu K, Liang CL, Chen HJ, Chen SD, Hsu HC, Liliang PC, et al. Injury severity and cell death mechanisms: effects of concomi tant hypovolemic hypotension on spinal cord ischemia-reperfus ion in rats. Exp Neurol 2004;185:120-32.
3. Blesch A, Tuszynski MH. Spinal cord injury: plasticity, regener ation and the challenge of translational drug development. Tre nds Neurosci 2009;32:41-7.
4. Li YN, Pan R, Qin XJ, Yang WL, Qi Z, Liu W, et al. Ischemic n eurons activate astrocytes to disrupt endothelial barrier via inc reasing VEGF expression. J Neurochem 2014;129:120-9.
5. Brockington A, Lewis C, Wharton S, Shaw PJ. Vascular endoth elial growth factor and the nervous system. Neuropathol Appl Neurobiol 2004;30:427-46.
6. Silverman WF, Krum JM, Mani N, Rosenstein JM. Vascular, glia l and neuronal effects of vascular endothelial growth factor in mesencephalic explant cultures. Neuroscience 1999;90:1529-4 1.
7. Sondell M, Lundborg G, Kanje M. Vascular endothelial growth f actor has neurotrophic activity and stimulates axonal outgrowth, enhancing cell survival and Schwann cell proliferation in the p eripheral nervous system. J Neurosci 1999;19:5731-40.
8. Bergeron M, Yu AY, Solway KE, Semenza GL, Sharp FR. Induc tion of hypoxia-inducible factor-1 (HIF-1) and its target genes following focal ischaemia in rat brain. Eur J Neurosci 1999;1 1:4159-70.
9. Francel PC, Long BA, Malik JM, Tribble C, Jane JA, Kron IL. Limiting ischemic spinal cord injury using a free radical scaven ger 21-aminosteroid and/or cerebrospinal fluid drainage. J Neu rosurg 1993;79:742-51.
10. Lian Jin H, Pennant WA, Hyung Lee M, Su S, Ah Kim H, Lu Li u M, et al. Neural stem cells modified by a hypoxia-inducible VEGF gene expression system improve cell viability under hyp oxic conditions and spinal cord injury. Spine (Phila Pa 1976) 2 011;36:857-64.
11. Karimi-Abdolrezaee S, Schut D, Wang J, Fehlings MG. Chondro

itinase and growth factors enhance activation and oligodendrocyte differentiation of endogenous neural precursor cells after spinal cord injury. PLoS One 2012;7:e37589.
12. Hwang do W, Kang JH, Jeong JM, Chung JK, Lee MC, Kim S, et al. Noninvasive in vivo monitoring of neuronal differentiation using reporter driven by a neuronal promoter. Eur J Nucl Med Mol Imaging 2008;35:135-45.
13. Schmechel DE, Brightman MW, Marangos PJ. Neurons switch from non-neuronal enolase to neuron-specific enolase during differentiation. Brain Res 1980;190:195-214.
14. Navarro V, Millecamps S, Geoffroy MC, Robert JJ, Valin A, Mallet J, et al. Efficient gene transfer and long-term expression in neurons using a recombinant adenovirus with a neuron-specific promoter. Gene Ther 1999;6:1884-92.
15. Maxwell PH, Pugh CW, Ratcliffe PJ. Inducible operation of the erythropoietin 3' enhancer in multiple cell lines: evidence for a widespread oxygen-sensing mechanism. Proc Natl Acad Sci U S A 1993;90:2423-7.
16. Wang GL, Semenza GL. General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. Proc Natl Acad Sci U S A 1993;90:4304-8.
17. Watanabe M, Sakaguchi M, Kinoshita R, Kaku H, Ariyoshi Y, Ueki H, et al. A novel gene expression system strongly enhances the anticancer effects of a REIC/Dkk-3-encoding adenoviral vector. Oncol Rep 2014;31:1089-95.
18. Xu ZL, Mizuguchi H, Ishii-Watabe A, Uchida E, Mayumi T, Hayakawa T. Optimization of transcriptional regulatory elements for constructing plasmid vectors. Gene 2001;272:149-56.
19. Forss-Petter S, Danielson PE, Catsicas S, Battenberg E, Price J, Nerenberg M, et al. Transgenic mice expressing beta-galactosidase in mature neurons under neuron-specific enolase promoter control. Neuron 1990;5:187-97.
20. Bhang SH, Kwon SH, Lee S, Kim GC, Han AM, Kwon YH, et al. Enhanced neuronal differentiation of pheochromocytoma 12 cells on polydopamine-modified surface. Biochem Biophys Res Commun 2013;430:1294-300.
21. Maier O, Bohm J, Dahm M, Bruck S, Beyer C, Johann S. Differentiated NSC-34 motoneuron-like cells as experimental model for cholinergic neurodegeneration. Neurochem Int 2013;62:1029-38.
22. Kim HA, Nam K, Lee M, Kim SW. Hypoxia/hepatoma dual specific suicide gene expression plasmid delivery using bio-reducib

le polymer for hepatocellular carcinoma therapy. J Control Release 2013;171:1-10.
23. Jin KL, Mao XO, Greenberg DA. Vascular endothelial growth factor: direct neuroprotective effect in in vitro ischemia. Proc Natl Acad Sci U S A 2000;97:10242-7.
24. Lee M, Lee ES, Kim YS, Choi BH, Park SR, Park HS, et al. Ischemic injury-specific gene expression in the rat spinal cord injury model using hypoxia-inducible system. Spine (Phila Pa 1976) 2005;30:2729-34.
25. Choi BH, Ha Y, Ahn CH, Huang X, Kim JM, Park SR, et al. A hypoxia-inducible gene expression system using erythropoietin 3' untranslated region for the gene therapy of rat spinal cord injury. Neurosci Lett 2007;412:118-22.
26. Jin H, Liu ML, Kim HA, Lee M, An S, Oh J, et al. Role of the oxygen-dependent degradation domain in a hypoxia-inducible gene expression system in vascular endothelial growth factor gene therapy. Spine (Phila Pa 1976) 2009;34:E952-8.
27. Lee M, Choi D, Choi MJ, Jeong JH, Kim WJ, Oh S, et al. Hypoxia-inducible gene expression system using the erythropoietin enhancer and 3'-untranslated region for the VEGF gene therapy. J Control Release 2006;115:113-9.
28. Choi UH, Ha Y, Huang X, Park SR, Chung J, Hyun DK, et al. Hypoxia-inducible expression of vascular endothelial growth factor for the treatment of spinal cord injury in a rat model. J Neurosurg Spine 2007;7:54-60.
29. An SS, Pennant WA, Ha Y, Oh JS, Kim HJ, Gwak SJ, et al. Hypoxia-induced expression of VEGF in the organotypic spinal cord slice culture. Neuroreport 2011;22:55-60.
30. Gerber HP, Condorelli F, Park J, Ferrara N. Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. J Biol Chem 1997;272:23659-67.
31. Oosthuyse B, Moons L, Storkebaum E, Beck H, Nuyens D, Brusselmans K, et al. Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. Nat Genet 2001;28:131-8.
32. Jin K, Mao XO, Batteur SP, McEachron E, Leahy A, Greenberg DA. Caspase-3 and the regulation of hypoxic neuronal death by vascular endothelial growth factor. Neuroscience 2001;108:351-8.
33. Volm M, Mattern J, Koomagi R. Inverse correlation between apoptotic (Fas ligand, caspase-3) and angiogenic factors (VEGF,

microvessel density) in squamous cell lung carcinomas. Antican cer Res 1999;19:1669-71.
34. Zhu Y, Jin K, Mao XO, Greenberg DA. Vascular endothelial gro wth factor promotes proliferation of cortical neuron precursors by regulating E2F expression. Faseb j 2003;17:186-93.
35. Iida K, Kawakami Y, Sone H, Suzuki H, Yatoh S, Isobe K, et al. Vascular endothelial growth factor gene expression in a retin al pigmented cell is up-regulated by glucose deprivation throu gh 3' UTR. Life Sci 2002;71:1607-14.
36. Wenger RH. Mammalian oxygen sensing, signalling and gene re gulation. J Exp Biol 2000;203:1253-63.
37. Dibbens JA, Miller DL, Damert A, Risau W, Vadas MA, Goodall GJ. Hypoxic regulation of vascular endothelial growth factor mRNA stability requires the cooperation of multiple RNA eleme nts. Mol Biol Cell 1999;10:907-19.
38. Springer ML, Chen AS, Kraft PE, Bednarski M, Blau HM. VEG F gene delivery to muscle: potential role for vasculogenesis in adults. Mol Cell 1998;2:549-58.

Abstract (in korean)

저산소 유도 신경 특이적 프로모터 벡터를 이용한 효율적인 혈관내피성장인자 발현

<지도교수 하 윤>

연세대학교 대학원 의과학과

윤 여 민

척수손상은 기계적인 1차 손상 이후 허혈성 조직환경이나 염증반응에 의해 세포사멸, 혈관손실, 탈수초 같은 2차 손상이 발생한다. 신경혈관 손상은 신경세포로의 산소전달을 감소시켜 산소에 대한 의존도가 높은 신경세포가 큰 타격을 받게 된다. 손상 후 이러한 허혈성 조직 환경은 손상을 회복하기 위해 신경 줄기세포에서 신경교세포로 분화가 이루어지지만 주로 성상교세포로 분화가 되고, 손상으로 인한 허혈성 환경에서의 성상교세포는 신경혈관의 기능을 약화시키게 된다. 때문에 척수손상을 치료하기 위해서 신경교세포보다 신경세포에서 선택적으로 많이 치료유전자를 발현시킬 수 있는 조절 가능한 벡터 시스템이 필요하다. 때문에 본

연구에서는 신경세포에 많이 존재하는 뉴런특이적에놀라아제를 프로모터로 이용하여 신경세포에서 유전자 발현효율을 높였다. 뉴런특이적에놀라아제는 분화된 신경세포에서 많이 존재하므로 신경세포가 분화됨에 따라 이 프로모터에 의한 유전자 발현이 증가하다는 것을 확인하였다. 또한 손상조직의 허혈성 조건에서 발현을 더 유도시키기 위해 저산소조건에서 많이 발현되는 에리스로포에틴을 인핸서로 이용하여 저산소조건에서 유전자가 더 발현됨을 확인하였다. 에리스로포에틴 인핸서와 뉴런특이적에놀라아제 프로모터를 결합한 벡터에서는 저산소 조건에있는 신경세포에서 유전자가 가장 많이 발현되었다. 이러한 유전자 발현 시스템을 확립한 뒤, 척수손상에 대한 치료유전자로서 혈관생성과 신경보호기능이 있는 혈관내피성장인자 또한 저산소환경의 신경세포에서 발현량이 증가함을 확인하였다. 저산소 유도 신경 특이적 유전자 발현 시스템을 이용해서 신경세포를 타겟으로 치료유전자의 기능을 효율적으로 증가시킴으로 인해 신경손상에 대한 다양한 유전자치료에 활용할 수 있을 것이라 생각된다.

핵심되는 말 : 신경 특이적 프로모터, 저산소 유도 유전자 발현, 뉴런 특이적에놀라아제, 에리스로포이틴, 혈관내피성장인자